Towards tissue engineering of the intervertebral disc

Peeters, M.

2017

document version
Publisher's PDF, also known as Version of record

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A DECREASE IN TONEBP EXPRESSION IN THE HYPEROSMOLAR INTERVERTEBRAL DISC

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In preparation

ABSTRACT

Intervertebral disc (IVD) degeneration can cause low-back pain, one of the major health problems in modern society. The vicious cycle of degeneration can be initiated by mechanical overloading. The osmotic properties of the disc are pivotal for withstanding loads. Upon overloading water is extruded from the IVD, changing the osmolality of the disc. In this study, the influence of high-osmotic culturing, which increases the osmolality of the IVD, on degenerative and osmosensitive markers is investigated in a near physiological set-up.

57 intact caprine intervertebral discs were cultured in an *ex vivo* bioreactor for >9 days under continuous simulated physiological axial loading. Control discs were cultured on regular medium. For the experimental groups, medium osmolality was increased at t=3 days by the addition of 26% (w/v) Poly-Ethylene-Glycol. To investigate recovery, medium osmolality was restored at t=6 days in the third group. At t=9, the expression of different genes, as well as the biochemical composition of the disc were analysed.

Increasing medium osmolality resulted in a decrease of disc height of 0.62 mm ($p<0.001$), and recovery of disc height during daily unloading was reduced by 65% ($p<0.001$). Analysis of gene expression profile showed a significant reduction in the expression of TonEBP and MMP-14 ($p=0.01$ and $p=0.003$, respectively) compared to control discs. Expression of other genes was not affected by hyperosmolality of the culture medium and no degenerative gene expression profile was observed. With the restoration of culture medium osmolality, both the biomechanical behaviour and gene expression were restored to control levels.

The observed decrease in the expression of TonEBP in reaction to the hyperosmolar environment contradicts the current literature. This contrast may be explained by the used model used, as complete IVDs were cultured in a physiological mechanical setting, contrasting to previous *in vitro* studies.

KEYWORDS

Intervertebral disc – osmolality – TonEBP/NFAT5 – cellular behaviour – disc degeneration – IVD biomechanics – rtPCR

INTRODUCTION

Low-back pain (LBP) is a leading cause of medical complaints in western society, with four out of five people suffering from severe LBP at least once in their life. Not only elderly but also the younger and working population is affected by LBP, resulting in high social-economic costs[1–3]. LBP is a multifactorial condition, but significant correlations have been found with intervertebral disc (IVD) degeneration[4,5]. It is well known that disc degeneration can be initiated by overloading[6–9]. However, the exact mechanotransductive pathways have not been elucidated. To prevent or stop degeneration, it may be crucial to understand this process.

The main function of the intervertebral disc is mechanical, as it provides flexibility and distributes loads over the spine. Crucial for this specific function is the extracellular matrix. However, this extracellular matrix is subject to damage and degeneration. Research shows that mechanical overloading can induce a vicious cycle of disc degeneration[6–10]. For example, three weeks of mechanical overloading in an organ culture system generated a catabolic cell response with increased inflammatory and remodelling markers in goat IVDs[6]. In response to overloading, water is extruded from the disc, leading to an increased shear force exerted on the cells and furthermore increasing the osmolality in the disc. However, mild mechanical loading is required for maintaining a healthy and functional extracellular matrix[11].

The mechanical loads on the intervertebral disc are opposed by an ability to attract and retain water. This is governed by the composition of the extracellular matrix. The centre of the IVD, the nucleus pulposus, is a gelatinous tissue composed of proteoglycans embedded with collagen type II[12]. The glycosaminoglycan (GAG) side chains of the proteoglycans are negatively charged and attract positive ions, generating a high osmotic pressure which pulls water into the NP. This osmotic pressure gives the intervertebral disc the ability to withstand the daily compressive loading. During daily activities, water is expelled from the disc due to the compressive loads, increasing the osmolality of the remaining fluid. When unloaded, the water flows back into the disc driven by the osmotic gradient[13–15]. Up to 25% of the disc's fluid is expelled and re-absorbed during the day, resulting in a diurnal change in osmolality of the IVD (430-550 mOsm)[16–18]. Disc height shows a comparable diurnal change[19,20]. In a previous study we have showed that an increase in osmolality of the IVD changes disc height and axial biomechanics, resembling disc degeneration[21].

Recent studies suggest tissue osmolality as a possible feedback mechanisms for mechanotransduction[22,23]. Tonicity-responsive Enhancer Binding Protein (TonEBP, also named NFAT5) was found to be activated by a shift to high osmotic culture medium, stimulating healthy matrix protein expression and synthesis[22–25]. TonEBP promotes the expression of

osmotolerance genes which facilitate ion exchange for compatible osmolytes, *e.g* sodium myo-inositol transporter (SMIT/Slc5a3), taurine transporters (TauT/ Slc6A6), sodium/ chloride coupled acid transporters, aquaporin channels and calcium binding proteins (S100a4)[23], thereby preventing damage to the cells' proteins and DNA, caused by the rapid uptake of cations in the initial response to hyperosmolality[26]. However, as the osmolality of the IVD is continuously interacting with the mechanical loading, the question arises whether results obtained from *in vitro* cell cultures -where the cells lose their connection with the extracellular matrix and, consequently, mechanotransductive pathways- can be translated to the *in vivo* situation[18,27]. In recent years, the introduction of *ex vivo* loaded disc culture systems[11,28–30], able to simulate physiological conditions for intact intervertebral discs, has allowed the study of IVD cells in their natural environment. This research shows that physiological loading is essential for cell viability and adequate extracellular matrix protein synthesis[11,28,29].

Therefore, we studied the effect of an increased osmolality of the culture medium on gene expression and biomechanical characteristics of the intervertebral disc. This will disturb the osmotic gradient resulting in water withdrawal from the disc, as occurs with mechanical overloading, increasing the osmolality of the IVD. However, the applied simulated physiological mechanical loading is left unchanged, and the intervertebral disc is kept intact. If the degenerative response, found with overloading[6], is mediated by the hyperosmolality, a similar response is expected in this study. Therefore, in this study, we investigated the cellular response of the goat IVD to hyperosmolality in an *ex vivo* culture system.

MATERIALS AND METHODS

Culturing intervertebral discs

Spines of skeletally mature 3-to-5-year-old Dutch milk goats were obtained from a local abattoir. The spines were rinsed with iodine solution, and soft tissue and posterior elements were removed under sterile conditions. 57 lumbar intervertebral discs (IVDs; T13L1 – L5L6) including adjacent cartilaginous endplates and a thin layer of vertebral bone were dissected using an oscillating surgical saw. Discs were subsequently washed in Phosphated Buffered Saline (PBS; Gibco) supplemented with Penicillin (10,000 units/ml), Streptomycin (10 mg/ ml) and Amphotericin B (25 µg/ml) (1% PSF; Sigma Aldrich). All discs were cultured in individual culture chambers in the custom build loaded disc culture system (LDCS) at 37 ^{o}C, 5% CO_2[6,11]. The first three days all IVDs were cultured in medium containing: Dulbecco's modified Eagle's medium (DMEM, Life technologies), supplemented with 10% Hyclone fetal bovine serum (FBS, Thermo Scientific), 1% PSF, 3.5 g/L glucose (Merck, final concentration 4.5 g/L), 25 mM HEPES buffer (Life Technologies) and 50 µg/ml ascorbate-2-phosphate

(Sigma Aldrich). After this initial culture period, the osmolality of the culture medium for the experimental group was increased by adding 26 % (w/v) poly-ethylene-glycol (PEG; MW 8000; Sigma Aldrich) to the culture medium. Culture medium was checked for osmolality by a freezing point osmometer (Osmomat 30, Gonotec, Germany); ~1050 mOsm for high osmotic culture medium and ~400 mOsm for iso-osmotic culture medium. Increasing the osmolality of the culture medium will reduce the osmotic gradient between the intervertebral disc and its environment and will, similar to mechanical overloading, withdraw water from the disc in order to restore the osmotic balance[31]. A total of ~55 ml medium was added to each individual culture chamber and refreshed every 3-4 days. In between medium refreshing, culture chambers were flushed with PBS supplemented with 1% PSF. All discs were subjected to a simulated physiological loading (SPL), consisting of a sinusoidal continuous axial dynamic loading resembling activities during the day and the night. The loading regime existed of 8 hours low dynamic load (50 ± 10 N at 1 Hz), followed by 16 hours of 30 minute blocks alternating in magnitude (130 ± 50N or 50 ± 10 N at 1 Hz) (Fig. 1)[11]. Discs were randomly assigned to one of the four experimental groups: control, high osmotic culture medium (PEG), and high osmotic culture medium with recovery (Recovery) (table 1).

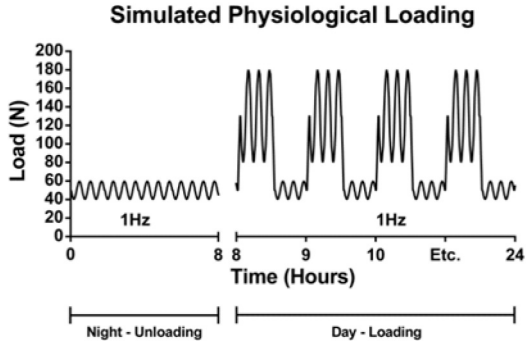

Figure 1 | Schematic view of the simulated physiological loading condition applied to all IVDs of the experiment cultured in the loaded disc culture system. The continued dynamic axial loading pattern is composed of 8 hours low dynamic load (sinusoidal; 50 ± 10 N at 1 Hz) resembling the night, followed by 16 hours of 30 minute blocks alternating in magnitude, resembling daily activities (sinusoidal; 130 ± 50N or 50 ± 10 N at 1 Hz).

Biomechanics

The applied forces to the IVDs and the displacement of the IVD were digitised at 100 Hz and customized programs in Matlab (version 2012b for Linux, Mathworks, Natick, MA, USA) were used for data analysis. Analysed outcome parameters are the overall disc height loss (mm) relative to the end of day three, and the daily recovery in disc height during the 8 hour low-load, a measure for the poro-elastic properties[20].

Table 1 | Overview of the different experimental groups and corresponding culture period on high osmotic culture medium (n=8 for the PEG group, n=6 for the Recovery rt-PCR group and n=5 for the Recovery histology – biochemistry group, the n of control groups is similar to the corresponding experimental group).

	Culture days												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Control													
PEG													
Recovery (rt-PCR)													
Recovery (Histology - Biochemistry)													

Iso-osmotic medium (~400 mOsm) High-osmotic medium (~1050 mOsm)

Real-time PCR

Samples of the nucleus pulposus (NP) and annulus fibrosus (AF) were obtained by the removal of one cartilage endplate and dissecting the tissue using a scalpel. Samples were immediately snap frozen in liquid nitrogen and stored at -80 ^{o}C. Tissue homogenisation, RNA isolation and subsequent real-time PCR analysis was performed as described by Peeters *et al.*[32]. In brief, tissues were homogenised with the MagNalyser, in 750 µl lysis buffer (RLT; Qiagen) in 4 runs of 40 sec at 6,500 rpm with in-between runs cooling on ice. Lysate was centrifuged, 3 minutes at 10,000 rpm, and supernatant was transferred to a new 1.5 ml tube. Total RNA was isolated with the RNeasy fibrous kit (Qiagen), including the DNase I treatment. Complementary DNA (cDNA) was synthesised with the Supercript VILO (Invitrogen) kit, in a 20 µl reaction containing 14 µl RNA, 4 μL VILO Reaction Mix and 2 μL SuperScript Enzyme Mix. Real-time PCR reactions were performed using the SYBRGreen reaction kit (Roche Diagnostics) in a LightCycler 480 system (Roche Diagnostics). LightCycler reactions were prepared in 10 µl total volume with 2 µl PCR-H2O, 0.5 µl forward primer (20 pM), 0.5 µl reverse primer (20 pM), 5 µl LightCycler Mastermix, to which 2 µl of five times diluted cDNA was added as a PCR template. Primer-sequences (Life Technologies) used for real-time PCR are listed in table 2, different anabolic, catabolic and osmosensitive genes were assessed. Tyrosine 3-mono-oxygenase/ tryptophan 5-mono-oxygenase activation protein, zeta polypeptide (YWHAZ) was used as a housekeeping gene and used as normalisation factor. Expression of the target genes was quantified with the fit point method in the LightCycler software, crossing points from a standard curve of six serial dilutions of DNA derived from each gene, were assessed. Relative gene expression is shown as the ratio between absolute expression of the target gene and normalisation factor (YWHAZ) of the same sample.

Table 2 | Primer sequences used for PCR

Gene	Oligonucleotide Sequence		Annealing temperature (˚C)	Amplicon lenth (bp)
YWHAZ	FW	5′ GATGAAGCCATTGCTGAACTTG 3′	56	229
	REV	5′ CTATTTGTGGGACAGCATGGA 3′		
ACAN	FW	5′ CAACTACCCGGCCATCC 3′	57	160
	REV	5′ GATGGCTCTGTAATGGAACAC 3′		
Col2b	FW	5′ AGGGCCAGGATGTCCGGCA 3′	56	195
	REV	5′ GGGTCCCAGGTTCTCCATCT 3′		
Col1	FW	5′ TCCAACGAGATCGAGATCC 3′	57	191
	REV	5′ AAGCCGAATTCCTGGTCT 3′		
TonEBP	FW	5′GCAGGAGGCACAATGAACC 3′	57	159
	REV	5′ TTGGCCTGGCTGACTTATGG 3′		
S100a4	FW	5′ GGTGTCCACCTTCCACAAGT 3	57	220
	REV	5′CACATCATGGCGATGCAG 3′		
Slc5a3	FW	5′ GGAGACAGCAGACATTGCCATA 3	57	251
	REV	5′ GCAGCAAGGCATTGAACTCC 3′		
MMP14	FW	5′ CTGAGATCAAGGCCAATGTTC 3′	56	206
	REV	5′CTCACGGATGTAGGCATAGG 3′		
MMP13	FW	5′ GGAGCATGGCGACTTCTAC 3′	56	208
	REV	5′ GAGTGCTCCAGGGTCCTT 3′		
ADAMTS4	FW	CATCCTACGCCGGAAGAGTC	57	278
	REV	GGATCACTAGCCGAGTCACCA		
ADAMTS5	FW	GTGGAGGAGGAGTGCAGTTTG	57	320
	REV	TTCAGTGCCATCGGTCACCTT		
IL1	FW	TGGAGCAACAAGTGGTGTTCT	57	270
	REV	GAGAGGTGCTGATGTACCAGTT		
IL6	FW	CTCTTCACAAGCGCCTTCAGT	57	248
	REV	GCCAGTGTCTCCTTGCTGTT		

Water content and quantitative biochemistry

Discs for biochemistry were dissected using a double-bladed handsaw to prevent water uptake, the mid-sagittal part was used for macroscopic and histological evaluation. From the lateral parts, samples were obtained for quantitative biochemistry. Samples were divided into nucleus pulposus (NP), inner annulus fibrosus (iAF) and outer annulus fibrosus (oAF). Tissue wet weight (WW) was measured, subsequently the tissue was freeze dried (Speedvac, Thermofisher, Waltham, USA) and dry weight (DW) was measured. Water content of the

samples was then defined as . The freeze-dried samples (~1 mg) were digested in a papain-digestion solution (5 mmol/L L-cysteine, 50 mmol/L EDTA, 0.1 M sodium acetate, pH titrated to 5.53 using 1 M NaOH and 3% (w/v) papain) at 60°C overnight. The digested solution was used to measure the sample's glycosaminoglycan (GAG) content using a 1.9-dimethyl-methylene blue (DMMB) assay (Biocolor Ltd) according to manufacturers' instructions. The remaining papain digestion solution was used to measure the total hydroxyproline (HYP) content, as a measure for total collagen content, through a dimethylamino-benzaldehyde (DMBA) hydroxyproline assay as previously described[33]. The measured amount of glycosaminoglycans and hydroxyproline was normalized to the dry weight for each sample.

Histology

Directly after dissecting the sagittal section of the IVD, it was photographed for macroscopic evaluation before fixation in 4% formalin. Sections were fixated for 10 days, decalcified in Kristensens fluid for a week, embedded in paraffin and cut into 3 µm sections. Matrix composition was analysed by staining with HE and Alcian Blue PAS and a histological grading scale for goat intervertebral disc degeneration was applied34.

Statistics

Prism 5 (GraphPad) was used to analyse the differences between the IVDs cultured on high osmotic medium and the discs cultured on standard medium. For the rt-qPCR, water content, histological evaluation and biochemistry independent Student's *t*-tests and one-way ANOVA's were used. For the biomechanical parameters, day 3, 6 and 9 were compared between the high- and low-osmotic groups (PEG and Control) using Student's t-tests. Each n represents one IVD, *p*-values < 0.05 were considered significant.

RESULTS

Biomechanics

After three days, all discs found a dynamic equilibrium for diurnal loading, with a loss of disc height during the 16 hours of high dynamic load, and an equal recovery of the disc height during the 8-hour night low load. The increase in medium osmolality after 3 days, by the addition of PEG to the culture medium, dramatically changes this equilibrium (Fig. 2). On average, the discs allocated to a PEG-group lose 0.62 (±0.20) mm of disc height in the 3 days after medium change, compared to 0.00 (±0.02) mm in the control group ($p<0.001$). The recovery of the disc height at night, a measure for the poro-elasticity 20, is reduced by 65 % on average ($p<0.001$). This implicates a major decrease of fluid inflow at night. However, after restoration of the medium osmolality in the recovery group, a rapid restoration of disc height is observed (Fig. 2). Disc height recovery at night was fully restored to original values

when measured at day 9 ($p<0.001$ compared to day 6, $p=0.72$ compared to day 3).

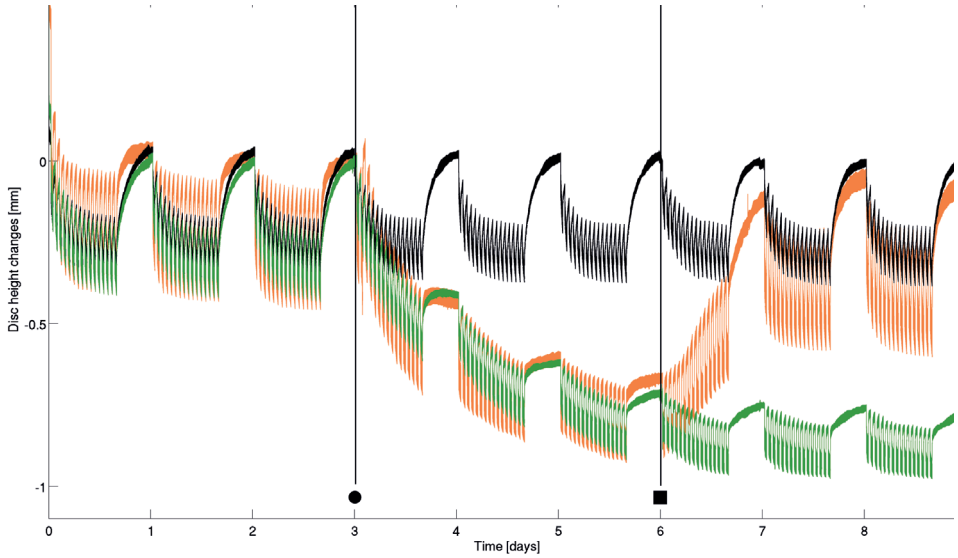

Figure 2| Typical examples of the changes in disc height over time for the different experimental groups. All groups were cultured on iso-osmotic culture medium during the first three days. Next (time point ●), the control group remained on iso-osmotic culture medium (black) and both PEG groups (green) (orange) were subjected to high-osmotic culture medium. At t=6 days (time point ▪), the culture medium of the recovery group (orange) was brought back to iso-osmotic values.

Observed changes in gene expression due to culturing on high osmotic culture medium

The most prominent effect in the gene expression analysis is a significant decrease of TonEBP expression in both the nucleus pulposus (NP) ($p=0.01$) and annulus fibrosus (AF) ($p=0.01$) of IVDs cultured on high osmotic culture medium for 6 days (Control vs PEG, Fig. 3). Aggrecan (ACAN) expression did not show significant differences after culturing on high osmotic medium ($p=0.14$ for NP and $p=0.21$ for AF, Fig. 3). Expression levels of the matrix degradation protein MMP-14 is significantly reduced in the NP of IVDs cultured on high osmotic medium ($p=0.003$), whereas no difference was found for the AF. The clear decrease in TonEBP expression was not observed in its target genes, S100a4 and Slc5a3, for both the NP and AF. Col2b expression could only be quantified for the NP cells but did not show any changes upon culturing on high osmotic culture medium ($p=0.39$). Expression of the remaining genes in our assay was detected, however, expression was too low to be reliable quantified. Upon restoration of the osmolality of the culture medium, no differences in gene expression between the control and recovery group were observed for both the NP and AF (Fig. 4). Although a trend towards reduction of TonEBP expression was found in the AF after recovery upon culture on high osmotic medium, this was not statistically significant ($p=0.14$).

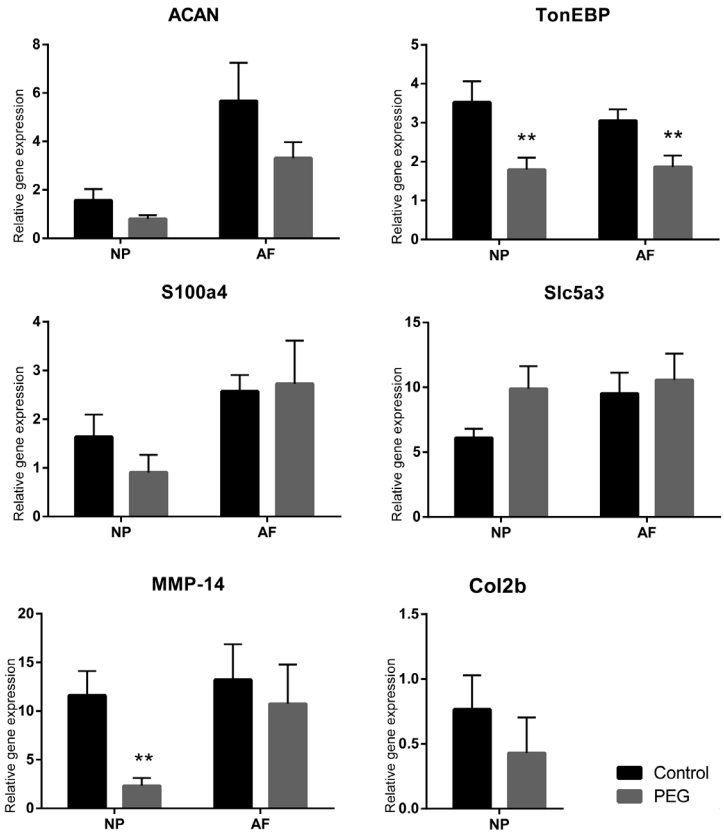

Figure 3| Shown are the relative gene expression of IVDs cultured on iso-osmotic culture medium (control, black) and high osmotic culture medium (PEG, dark grey) for both the nucleus pulposus (NP) and annulus fibrosus (AF) measured after 6 days. ACAN; aggrecan, TonEBP; Tonicity Enhancer Binding Protein, S100a4; Calcium binding protein A4, Slc5a3: Sodium/myo-inositol cotransporter 5a3, MMP14: Matrix metalloproteinase 14, Col2b; Collagen type IIb. All genes are expressed relative to the housekeeping gene YWHAZ. Data is represented as mean ± SEM, ** indicate p≤0.01 and is determined for Control versus High Osmotic, n=8. All genes are expressed relative to the housekeeping gene YWHAZ.

Biochemistry and histology

The water content of the both the NP, inner AF and outer AF did not show any differences between the control discs cultured on iso-osmotic culture medium and the disc cultured on high osmotic medium for three days followed by recovery on iso-osmotic medium (p=0.86, p=0.49 and p=0.23, respectively, figure 5). The biochemical composition of the IVD, as measured by the glycosaminoglycan (GAG) and the hydroxyproline (HYP) content, also did not show any differences as. Furthermore, no differences in both the macroscopic and histological evaluation were observed (Fig. 6). None of the IVDs were scored more than mildly degenerated and bulging of the annulus fibres was not observed in any of the discs.

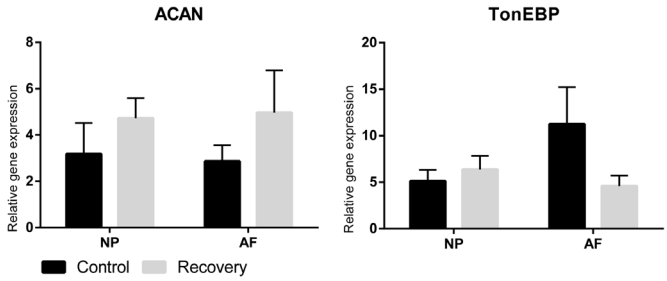

Figure 4 | Shown are the relative gene expression after restoration of culture medium osmolality of IVDs cultured on iso-osmotic culture medium for the complete period (control, black) and IVDs cultured on high osmotic culture medium the three days before restoration (Recovery, light grey) for both the nucleus pulposus (NP) and annulus fibrosus (AF). ACAN; aggrecan, TonEBP; tonicity enhancer binding protein. Data is represented as mean ± SEM, n=6. All genes are expressed relative to the housekeeping gene YWHAZ.

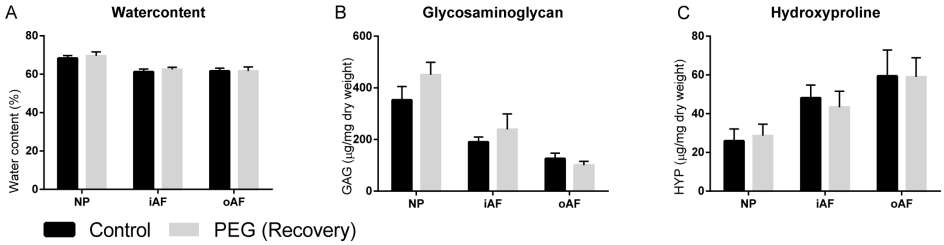

Figure 5| Overview of the different biochemical parameters for the nucleus pulposus (NP), inner annulus fibrosus (iAF) and outer annulus fibrosus (oAF) after restoring the osmolality of the culture medium back to iso-osmotic conditions. **A|** watercontent, **B|** Glycosaminoglycan (GAG) content, **C|** Hydroxyproline (HYP) content as a measure for collagen. No differences were found.

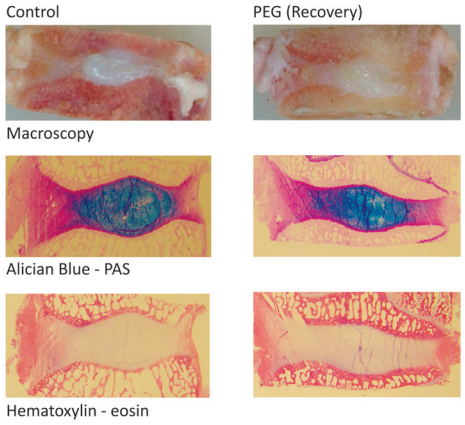

Figure 6| Overview of typical IVDs of the control and Recovery group (cultured on high osmotic medium for three days) used for the macroscopic (top) and histological (bottom two). Upon applying the goat IVD scoring system34, no statistical significant differences were found.

DISCUSSION

In this study, we investigated the effect of increasing the culture medium osmolality on both the biomechanical and cellular behaviour of the intervertebral disc. By increasing the culture medium osmolality, we found a clear decrease in disc height and a reduced recovery during unloading of the disc. These effects were reversible, as biomechanical behaviour was fully restored after restoring medium osmolality back to normal. Increasing the osmolality of the culture medium did not cause a change towards a catabolic, i.e. degenerative, gene expression, but a significant decrease in the TonEBP and MMP-14 expression was observed. Also, these changes were reversible. Reducing the osmotic gradient did not damage the extracellular matrix of the IVD in this time frame, as measured by both biochemistry and histological evaluation.

Although the loss of water and disc height and recovery of the IVDs cultured in hyperosmolar medium resembles degeneration, the decrease in MMP-14 expression and unchanged expression of aggrecan shows that no degenerative gene expression was induced. This is in contrast to mechanical overloading, which resulted in a degenerative gene expression profile in previous research[6]. Our results indicate that direct mechanical cues, rather than osmotic changes, induce a degenerative cell response. By increasing the osmolality of the culture medium, the osmotic gradient between the intervertebral disc and its environment was disturbed. Water was withdrawn from the disc, which increased the osmolality in the intervertebral disc. Therefore, an increase of TonEBP was expected based on the results from previous studies[22–24]. The contradictory finding of a decrease in TonEBP expression is remarkable and questions whether our *ex vivo* work or earlier *in vitro* experiments reflect a more physiological setting when investigating cellular responses

Within each diurnal cycle, up to 25% of the water in the IVD is expelled and reabsorbed resulting in large changes in the osmotic pressure of the disc; upon overloading even more water is extruded and the osmolality in the IVD increases further[6,16,18]. In this study IVDs were cultured in medium with increased osmolality (~1050 mOsm) by the addition of Poly-Ethylene-Glycol. This molecule is too large to enter the disc, and is also not charged and therefore no attractor of ions, in contrast to NaCl[35]. The decrease in disc height and reduced recovery during unloading of the discs cultured on high osmotic medium are similar to the changes we previously found in our research group. These changes resemble the changes seen in degenerated human discs[20,36], and are presumably caused by a decrease in water content of 8% for the NP due to hyperosmolar loading, as found previously[21]. Both disc height and the recovery during unloading were completely restored to control values upon restoring medium osmolality to normal values. This shows that the IVDs are not damaged before or during the culture period, which is confirmed by the biochemical and histological

evaluation. Both the endplate and annulus fibrosus were intact and did not show any changes compared to control IVDs.

In contrast to our findings, an increase in TonEBP expression of NP cells and human articular chondrocytes was found previously in response to increased osmolality of the medium [22,24]. However, these studies all involved *in vitro* 2D cultured cells, using 2D expanded cells with the risk of dedifferentiation, changes in gene expression, lost connection with the extracellular matrix and without the application of loading[27,37–39]. In contrast to the decreased TonEBP expression, we observed no effects in the downstream target genes of TonEBP, S100a4 and Slc5a3. In research by van der Windt *et al.*, the TonEBP target genes S100a4 and Slc6a12 showed an increased expression upon culturing chondrocytes in hyperosmotic conditions[24]. In this particular study, the highest osmolality is 380 mOsm, which is suggested physiological for chondrocytes. In rat NP cells, an increase in osmolality also induced an increase in the expression of the target genes (*e.g.* Taut (Slc6A6), SMIT (Slc5a3) and BGT-1)[22]. However, this was only observed when the osmolality was increased from 330 mOsm to 400 mOsm, a further increase (500 mOsm) had no additional effect. As the physiological osmolality of the healthy IVD is ~430 mOsm, the isotonic medium of 330 mOsm is considered hypo-osmotic[17]. Culturing NP explants in a hyperosmolar environment (~570 mOsm) is used to prevent the explants from swelling and preserve the matrix specific composition[40]. Whereas the mentioned studies showed an increased TonEBP expression after ~5 days of culture on hyperosmolar medium, only a transient increase in TonEBP expression after 6 hours was found for human NP cells in response to increased osmolality[25]. In renal MDCK cells it is shown that the increased expression on TonEBP caused by hyperosmolality is transient, peaking after 4 hours and followed by a decline[41,42]. In literature, a decrease in TonEBP expression of cartilaginous cells upon hyperosmolality has not been reported to our knowledge.

TonEBP also promotes aggrecan expression, thereby allowing adaptation to the hyperosmolar environment by regulating the expression and synthesis of aggrecan[22]. Although the reduction of TonEBP expression in our experiment may indicate a downregulation of aggrecan, we did not find any changes in aggrecan expression upon hyperosmolality. In the study by Wuertz *et al.*, an increase in aggrecan expression of human NP cells, in a 3D culture in collagen type I hydrogel, was found with an increasing osmolality (300 – 400 – 500 mOsm). However, when hydrostatical load was applied to the 3D cultures, aggrecan expression was decreased at higher osmolality (400 and 500 mOsm)[43]. The decrease in MMP-14 expression we found in our study is in correspondence with the decrease in MMP-3 expression of bovine NP cells and the decrease in the expression of MMP 13 and 14 in human NP cells upon high osmotic conditions[25,44].

Besides osmotic activation, it is suggested that TonEBP can also be affected via mechanical

deformation as shown by α1β1 and α6β4 integrin and stretching activation of TonEBP[45,46]. This is, however, only shown for kidney and vascular smooth muscle cells. To our knowledge, this is the first study in complete organs, describing the course of TonEBP expression as a result of large variations in osmolality.

This study is limited by the fact that gene expression was only analysed at a single time point. For future studies it would be interesting to include a broad time spectrum investigating changes on the short term (i.e. 4 to 6 hours), but also on the longer term (>28 days) to see if a degenerative gene expression profile and damage to the extracellular matrix will occur. Even though we used complete IVDs cultured in the loaded disc culture system for our study, it remains a model for the *in vivo* situation. Moreover, the IVDs used are obtained from healthy goats and did not show any signs of degeneration prior to the experiment.
In conclusion, by increasing the osmolality of the IVD, we induced changes in biomechanical behaviour of the disc similar to degenerative behaviour. This caused a decrease in the expression of TonEBP, contrasting to current literature, possibly indicating that results from *in vitro* cell cultures cannot be translated one-on-one to physiological circumstances. This discrepancy could be a reason why promising results from many *in vitro* studies never made it to the clinic. Our results do not give an indication that the degenerative cascade is initiated via osmotic signalling pathways. A better insight in the osmotic-related mechanotransductive pathways is of great importance to prevent or stop intervertebral disc degeneration. Furthermore, it can provide relevant information for tissue engineering strategies with osmotic hydrogels aiming for regeneration of the IVD.

ACKNOWLEDGEMENTS

The authors acknowledge the support from Paul Sinnige and Klaas-Walter Meijer for their support with the practical work.

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